

Crustacean Hyperglycemic Hormone Family: Old Paradigms and New Perspectives¹

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SYNOPSIS. I present an overview of recent research on the isolation and characterization of members of the crustacean hyperglycemic hormone (CHH) neuropeptide family. Members of this arthropod-specific family include CHH, molt-inhibiting hormone (MIH), vitellogenesis-inhibiting hormone (VIH), and mandibular organ-inhibiting hormone (MOIH). There are two subfamilies of this neuropeptide group, based upon the presence or absence of a C-terminal CHH precursor-related peptide. There are also sequence motif differences between these subfamilies. Most of the peptides comprising this neuropeptide family are synthesized and released by the eyestalk X-organ/sinus gland complex. Recent experiments have demonstrated the presence of extra-eyestalk cells that produce CHH and the assignment of additional functions to this hormone family.

INTRODUCTION: CHARACTERIZATION OF CHH AND MIH

In the field of crustacean endocrinology, one of the most interesting observations is the multi-functionality of the novel arthropod neuropeptide family related to the crustacean hyperglycemic hormone (CHH). I will refer to this group of related hormones somewhat arbitrarily as the CHH family simply because CHH was the first member of the family to be sequenced. The purpose of this paper is to present an overview of work on various members of this hormone family with an emphasis on the more recently published work. It is not meant to be comprehensive; rather, it has a personal focus on work from my laboratory.

Unlike insects, crustaceans appear to regulate their molting glands with a neuropeptide inhibitor. Almost a century ago, Zeleny (1905) removed the eyestalks from the fiddler crab, *Uca pugilator*, and observed a dramatic shortening of the molt interval (length of time between molts). This observation led to the postulation of a humoral factor in the eyestalks that normally acts as

a molt-inhibiting hormone (MIH). Other observations were made that the eyestalks were also the location of a humoral factor that regulated blood glucose concentrations. Abramowitz *et al.* (1944) observed that injections of eyestalk extracts resulted in the elevation of hemolymph glucose in crabs. Because of the observed hyperglycemic effect, the responsible factor was called CHH.

Based upon detailed microscopic studies, a neurohemal organ was described in those decapod crustaceans examined (Bliss and Welsh, 1952; Passano, 1953). This neurohemal organ is called the sinus gland. It consists of the enlarged terminals of a group of neurosecretory neurons collectively called the X-organ. The sinus gland serves as a storage and release site for the neurosecretory granules produced by the X-organ neurons. It has been presumed that different neurons synthesize and secrete the different hormones (MIH and CHH). Much of the early physiological and histological work on MIH and CHH has already been reviewed (Keller *et al.*, 1985; Kleinholz and Keller, 1979; Skinner, 1985; Keller and Sedlmeier, 1988; Chang, 1989; Keller, 1992).

Since our laboratory had been working on ecdysteroid (molting hormone) secretion for several years, we became interested in the regulation by MIH. I collaborated with Robert Newcomb, who had perfected HPLC techniques for the separation and

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quantification of sinus gland peptides from the crab *Cardisoma carnifex* (Newcomb, 1983; Newcomb *et al.*, 1985). This species has unusually large eyestalks and sinus glands. Using HPLC, we purified extracts of lobster (*Homarus americanus*) sinus glands and observed that only a single fraction had MIH activity based on the activity of injected fractions to increase the molt interval and to decrease circulating titers of ecdysteroids (Chang *et al.*, 1987).

Shortly thereafter, Rainer Keller's group published the first amino acid sequence of a CHH (Kegel *et al.*, 1989). Their experimental animal was the green crab *Carcinus maenas*. This 72 amino acid peptide did not belong to any previously known neuropeptide families. It has a pyroglutamate at the N-terminus and a valine-amide at the C-terminus. It has six cysteines and Kegel *et al.* (1989) were able to determine the locations of the three disulfide bonds from analyses of proteolytic digests.

We continued our studies on *H. americanus* MIH (Hoa-MIH) in collaboration with Glenn Prestwich and partially sequenced a peptide that had both MIH and CHH activity (Chang *et al.*, 1990). It was clearly related to *C. maenas* CHH. Using molecular methods, Van Herp and colleagues independently determined the sequences of two lobster CHHs (CHH-A and CHH-B; Tensen *et al.*, 1991). Subsequent work in our laboratory has verified the identity of Hoa-MIH with CHH-A. The perplexing question, that still remains unresolved, is how can such vastly different functions—molt-inhibition and elevation of hemolymph glucose—be precisely regulated by the same peptide? As will be discussed below, this problem becomes even more complex.

MORE MEMBERS JOIN THE FAMILY

Also working on *H. americanus*, Soyez's group published the sequence of another member of the CHH family that was able to inhibit vitellogenesis in a heterologous system (the shrimp *Palaemonetes varians*; Soyez *et al.*, 1991). Based upon their bioassay, they named this peptide vitellogenesis-inhibiting hormone (VIH). Its function in the lobster, however, has not yet been

determined. Since the initial identification of CHH, MIH, and VIH physiological activities attributable to this family, several other functions have been ascribed to these related peptides. Working with the locust, *Schistocerca gregaria*, Phillips' group identified and characterized a family member that mediates ion transport in the gut (Audsley *et al.*, 1992). Based upon its amino acid sequence, this ion transport peptide is definitely a member of the CHH family (Meredith *et al.*, 1996) and demonstrates that the CHH family is not restricted to crustaceans.

Recent evidence indicates that CHH itself also regulates ion and water balance in crustaceans. Spanings-Pierrot *et al.* (2000) observed that CHH from the crab *Pachygrapsus marmoratus* was able to increase the transepithelial potential difference and sodium influx of isolated gills. In *C. maenas*, Chung *et al.* (1999) observed a dramatic rise (several orders of magnitude) and peak in circulating CHH just at ecdysis. My laboratory has made similar unpublished observations in *H. americanus* in collaboration with Keller. Chung *et al.* (1999) concluded that this surge in CHH mediates the completion of ecdysis via the rapid uptake of water.

Another function has been attributed to the CHH family. From the crab *Cancer pagurus*, two novel peptides were isolated and sequenced. Both peptides inhibited the synthesis of the terpenoid methyl farnesoate by the mandibular organ *in vitro*. These peptides were named mandibular organ-inhibiting hormones (MOIHs). Although the peptides are definitely members of the CHH family, they did not have CHH activity (Wainwright *et al.*, 1996). In the crab *Libinia emarginata*, Laufer's group identified a peptide that had the ability to inhibit synthesis of methyl farnesoate by mandibular organs *in vitro* (Liu and Laufer, 1996; Liu *et al.*, 1997). This MOIH also had hyperglycemic activity.

The existence of peptides with multiple biological activities is further illustrated by the work of Khayat *et al.* (1998). They isolated a group of seven distinct peptides from the shrimp *Penaeus japonicus*. Six of the peptides had hyperglycemic activity,

three had molt-inhibiting activity, and all of the peptides were able to inhibit the incorporation of radiolabeled methionine into ovarian fragments *in vitro*.

I have compiled a table that contains most of the current members of the CHH family (Table 1). For sequence data and alignments, see Lacombe *et al.* (1999).

GENE STRUCTURE AND EXPRESSION

The use of molecular techniques has permitted a number of studies on the structure and expression of the CHH family genes. In *C. maenas* (Weidemann *et al.*, 1989) and the crayfish *Orconectes limosus* (De Kleijn *et al.*, 1994a), the CHH preprohormone has a 26 amino acid signal peptide. At the C-terminus, the signal peptide is followed by a 38 amino acid CHH precursor-related peptide (CPRP) in *C. maenas*. The CPRP is 33 amino acids long in both *O. limosus* and *H. americanus* (Tensen *et al.*, 1991; De Kleijn *et al.*, 1995). The actual CHH peptide is located at the C-terminal end of the CPRP.

The function of the CPRP is unknown. A universal hypothesis for its function is complicated by the observation that CPRP is not present in either *H. americanus* prepro-VIH or the majority of the prepro-MIH peptides. Thus two major subgroups of the family arise. These subgroups are CHH and MIH/VIH/MOIH and are also distinguished by different motifs of sequence data (Lacombe *et al.*, 1999). Caution must be exercised, however, since the nomenclature for several members of the family is based upon a circular argument. In other words, a newly sequenced peptide is called a MIH and placed into the MIH/VIH subgroup based upon its sequence similarity to previously published members of the family and not necessarily because of its biological activity.

Two distinct genes for *O. limosus* CHH have been characterized by De Kleijn *et al.* (1994a). The genes code for preprohormones that differ slightly in their signal peptides and their CPRPs, but are identical in their CHH coding regions. The two genes are expressed in different amounts in different individuals. Since both genes code for the identical CHH peptide, the two ob-

served CHH isoforms must be due to post-translational modification. It is not known if this post-translational modification is an isomerization of a gene-encoded L-amino acid to a D-amino acid as described below. In contrast to the ratio of the mRNAs from the two CHH genes, the ratio of the two CHH peptide isoforms was similar in individual crayfish (De Kleijn *et al.*, 1994a).

De Kleijn *et al.* (1995) also sequenced genes coding for CHH-A and CHH-B in *H. americanus*. They observed DNA sequence variations between the two genes that resulted in amino acid substitutions in the signal peptides, the CPRPs, and the CHH peptides.

Recent research indicates that some of the isoforms seen in the CHH family may be due to isomers of specific amino acid residues. Soyez *et al.* (1994) observed two isoforms of both CHH-A and CHH-B. They determined that these isoforms were due to the presence of either the L- or D-configuration of phenylalanine at the third position (Phe³). The different isoforms resulted in differential hyperglycemic responses. [L-Phe³]CHH-A produced maximal hyperglycemia after 2 hr; [D-Phe³]CHH-A produced its maximal effect after 3–4 hr. The levels of hyperglycemia produced by the two isoforms, however, were the same. Release rates of each of the isomers was similar under basal conditions (Ollivaux and Soyez, 2000).

Yasuda *et al.* (1994) observed a similar phenomenon in the CHH of *Procambarus clarkii* (Prc-CHH). Prc-CHH-I has an L-Phe³, while Prc-CHH-II has a D-Phe³. The two isoforms had similar hyperglycemic activities but Prc-CHH-II had a 10-fold greater MIH activity in an *in vitro* Y-organ assay. The authors concluded that this isomerism could explain the differential biological effects of CHH (hyperglycemia and molt-inhibition).

There appear to be multiple copies of some members of the peptide family. There are at least two copies of the MIH gene and 3–10 copies of the MOIH gene in *C. pagurus* (Tang *et al.*, 1999; Lu *et al.*, 2000).

Sufficient sequence data have now been accumulated to permit the construction of a dendrogram of the family and the identi-

TABLE 1. *Listing of species, putative activities, and references for sequenced neuropeptides of the CHH family.*

Arv-CHH	Martin <i>et al.</i> , 1993
Arv-VIH	Greve <i>et al.</i> , 1999
Canm-MIH	Umpfrey <i>et al.</i> , 1998
Cap-CHH-I, II	Chung <i>et al.</i> , 1998
Cap-MIH	Chung <i>et al.</i> , 1996
Cap-MOIH-I, II	Wainwright <i>et al.</i> , 1996
Carm-CHH	Kegel <i>et al.</i> , 1989; Weidemann <i>et al.</i> , 1989
Carm-MIH	Webster, 1991; Klein <i>et al.</i> , 1993
Cas-MIH	Lee <i>et al.</i> , 1995
Chf-MIH	Chan <i>et al.</i> , 1998
Hoa-CHH-A/MIH	Chang <i>et al.</i> , 1990; Tensen <i>et al.</i> , 1991; De Kleijn <i>et al.</i> , 1995
Hoa-CHH-B	Tensen <i>et al.</i> , 1991; De Kleijn <i>et al.</i> , 1995
Hoa-VIH-I	Soyez <i>et al.</i> , 1991
Hoa-VIH-II	Soyez <i>et al.</i> , 1994; De Kleijn <i>et al.</i> , 1994b
Jal-CHH-I	Marco <i>et al.</i> , 1998
Jal-CHH-II	Marco <i>et al.</i> , 2000
Lam-LMWP	Gasparini <i>et al.</i> , 1994
Lie-MOIH	Liu <i>et al.</i> , 1997
Mar-CHH	Sithigorngul <i>et al.</i> , 1999
Mee-CHH-A	Gu and Chan, 1998a
Mee-CHH-A	Gu <i>et al.</i> , 2000
Mee-MIH	Gu and Chan, 1998b
Orl-CHH	Kegel <i>et al.</i> , 1991; De Kleijn <i>et al.</i> , 1994a
Pej-CHH/SGP-III	Yang <i>et al.</i> , 1995; Ohira <i>et al.</i> , 1997a
Pej-MIH/SGP-IV	Yang <i>et al.</i> , 1996; Ohira <i>et al.</i> , 1997b
Pem-SGP-I-V	Davey <i>et al.</i> , 2000
Pes-CHH	Huberman <i>et al.</i> , 2000
Pev-MIH-like	Sun, 1994
Prb-CHH-I	Huberman <i>et al.</i> , 1993
Prb-CHH-II	Aguilar <i>et al.</i> , 1995
Prb-MIH	Aguilar <i>et al.</i> , 1996
Prc-CHH-I, II	Yasuda <i>et al.</i> , 1994
Prc-MIH	Nagasawa <i>et al.</i> , 1996
Scg-ITP	Meredith <i>et al.</i> , 1996

Abbreviations for species: Arv: *Armadillidium vulgare*; Canm: *Cancer magister*; Cap: *Cancer pagurus*; Carm: *Carcinus maenas*; Cas: *Callinectes sapidus*; Chf: *Charybdis feriatus*; Hoa: *Homarus americanus*; Jal: *Jasus lalandii*; Lam: *Latrodectus mactans tredecimguttatus*; Lie: *Libinia emarginata*; Mar: *Macrobrachium rosenbergii*; Mee: *Metapenaeus ensis*; Orl: *Orconectes limosus*; Pej: *Penaeus japonicus*; Pem: *Penaeus monodon*; Pes: *Penaeus schmitti*; Pev: *Penaeus vannamei*; Prb: *Procambarus bouvieri*; Prc: *Procambarus clarkii*; Scg: *Schistocerca gregaria*. Abbreviations for activities: CHH: crustacean hyperglycemic hormone; ITP: ion-transport peptide; LMWP: low molecular weight protein; MIH: molt-inhibiting hormone; MOIH: mandibular organ-inhibiting hormone; SGP: sinus gland peptide; VIH: vitellogenesis-inhibiting hormone. (Note that there is a recommendation that the genus name *Litopenaeus* be substituted for *Penaeus* for *P. schmitti* and *P. vannamei* and *marsupenaeus* for *P. japonicus*; Perez-Farfante and Kensley, 1997).

cation of conserved sequence motifs (Lacombe *et al.*, 1999). The arrangement and expression of genes of the CHH family have been reviewed in greater detail by De Kleijn and Van Herp (1995) and Van Herp (1998).

As more DNA probes for the peptide family become available, researchers will be able to address the factors that influence transcription of the genes. Some studies have been published indicating that different levels of peptide transcripts can be mea-

sured throughout the molt cycle (Reddy *et al.*, 1997; Lee *et al.*, 1998). Whether these differences during the molt cycle are due to differential transcription rates (as opposed to differential degradation or processing) remains to be determined.

Recent progress has been made in the expression of recombinant CHH peptides. Using *Escherichia coli*, MIH from *P. japonicus* (Ohira *et al.*, 1999) and from *Metapenaeus ensis* (Gu *et al.*, 2001) were successfully expressed with biological activity.

Other expression systems have used the yeast *Pichia pastoris* (Sun, 1997) and insect cells with a baculovirus (Watson, 1999). The availability of large quantities of biologically active peptides will certainly be important for more detailed physiological studies.

IMMUNOLOGICAL ASSAYS FOR CHH

Several rapid immunological assays for the CHH family have greatly assisted in studies into the localization and physiology of the member peptides. Radioimmunoassays (RIAs) were developed for the CHHs of *C. maenas*, *O. limosus*, and *C. pagurus* (Keller, 1988; Webster, 1996). These RIAs, however, were difficult to conduct and required radiolabeled hormone. More recently, sensitive enzyme-linked immunosorbent assays (ELISAs) were developed for the crab *C. carnifex* (Keller *et al.*, 1994) and *H. americanus* (Chang *et al.*, 1998). My laboratory has used the ELISA to quantify circulating levels of CHH in lobsters following environmental stress, such as hypoxia (Chang *et al.*, 1998), and parasitic infection (Stentiford *et al.*, 2001).

CHH PEPTIDES OUTSIDE OF THE X-ORGAN/ SINUS GLAND

There are some limited reports indicating the synthesis and release of CHH family peptides from non-eyestalk locations of the central nervous system (Keller *et al.*, 1985; De Kleijn *et al.*, 1995; Dirksen and Heyn, 1998; Chang *et al.*, 1999b; Gu *et al.*, 2000). In addition, our laboratory reported small, but significant concentrations of CHH immunoreactivity in the hemolymph of lobsters that had been eyestalk-ablated for over one year (Chang *et al.*, 1998). These ablated lobsters also displayed an elevation in hemolymph CHH following emersion-induced hypoxia. Based on these observations, we conducted a study to examine the neurons-of-origin of these peptides. Using ELISA, we assayed various portions of the lobster thoracic nervous system (Chang *et al.*, 1999a). The highest levels were found in the second roots emerging from thoracic ganglia 1–5 (up to 200 fmol/mg tissue).

These thoracic second roots contain cells that were described several decades ago as

being neurosecretory based upon their morphological and physiological properties (Evans *et al.*, 1976; Konishi and Kravitz, 1978; Livingstone *et al.*, 1981). In collaboration with Edward Kravitz and Barbara Beltz, we observed that many of the cells in the second roots stained positively for CHH (Fig. 1). *In vitro*, these cells and a pair of cells in the subesophageal ganglion release CHH in a calcium-dependent manner when depolarized with elevated potassium (Chang *et al.*, 1999a).

We are uncertain if this non-eyestalk CHH is identical to the lobster X-organ/sinus gland CHH previously sequenced. We are also uncertain about the function of this extra-eyestalk CHH. Based upon measurements of hemolymph CHH, the amount of extra-eyestalk CHH is unlikely to contribute significantly to glucose regulation at the organismal level. However, it could mediate a localized regulation of cellular glucose metabolism. For example, under periods of stress, localized release of CHH could be important in meeting elevated metabolic requirements of neural cells. It is also possible that the extra-eyestalk material could serve a neuromodulatory role as has been ascribed to other “classical” crustacean eyestalk hormones, such as red pigment-concentrating hormone (RPCH).

Extra-eyestalk CHH could also mediate functions that are distinct from glucose metabolism. Recent publications from Webster's and Dirksen's laboratories describe paraneurons in distinct areas of the crab (*C. maenas*) foregut and hindgut (Chung *et al.*, 1999; Webster *et al.*, 2000). These cells only express CHH during ecdysis and these cells are likely the source of the CHH surge during ecdysis that mediates water uptake.

PROSPECTS

I think that the field of crustacean neuroendocrinology is entering an exciting period that will depend upon a range of old (organ culture, histology) and new (molecular) experimental techniques. It will be necessary to use homologous bioassays on each of the purified hormones to determine the range and magnitude of activities (*e.g.*, is the primary function of an identified CHH actually the regulation of hemolymph

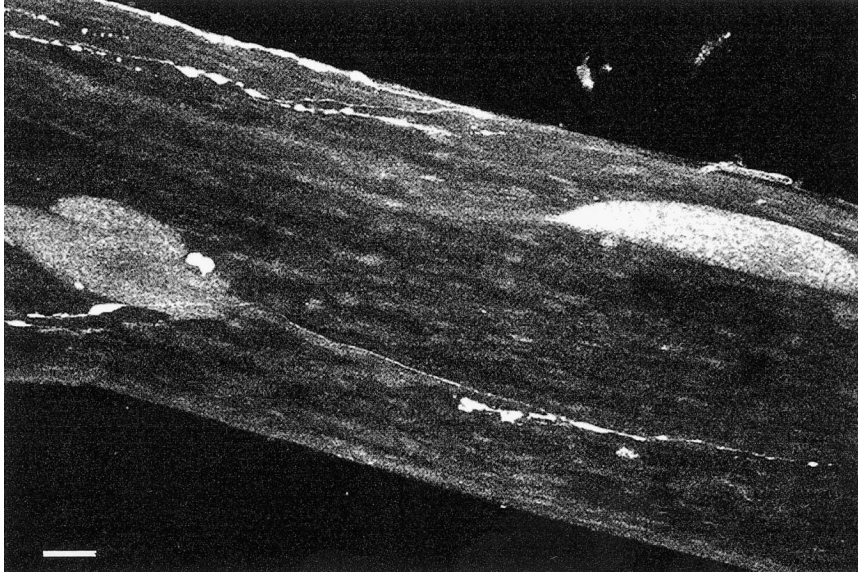


FIG. 1. A confocal microscopic projection of 16 collected (0.2 μm sections) images of neurosecretory cells near the bifurcation of the second root of the subesophageal ganglion of a lobster. It also shows the laterally projecting axon of the lower cell. The tissue was fixed and stained with cyanine dye-conjugated IgG made against *H. americanus* CHH-A. Scale bar = 5 μm . Details of the method have been described (Chang *et al.*, 1999a).

glucose?) Sorting out the diversity of functions of the CHH family will ultimately depend upon further experiments on the temporal and tissue-specific aspects of gene expression. In order to identify target tissues, characterization of specific hormone receptors will also be necessary.

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